High expression of LY6E is an independent prognostic factor of colorectal cancer patients

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Abstract. Colorectal cancer (CRC) is common cancer worldwide, and the 5-year relative survival rate of CRC patients with distant metastasis is as low as 14%. Therefore, identifying markers of CRC is important for the early detection of CRC and applying appropriate treatment strategies. The lymphocyte antigen 6 family (LY6 family) is closely related to the behavior of various cancer types. Among the LY6 family, the lymphocyte antigen 6 complex, locus E (LY6E), which is specifically highly expressed in CRC. Hence, the effects of LY6E on cell function in CRC and its role in CRC recurrence and metastasis were investigated. Reverse transcription-quantitative PCR, western blotting and *in vitro* functional studies were carried out using four CRC cell lines. Immunohistochemical analysis of 110 CRC tissues was performed to explore the biological functions and expression patterns of LY6E in CRC. LY6E was overexpressed CRC tissues compared with that in adjacent normal tissues. High expression of LY6E in CRC tissues was an independent prognostic factor of worse overall survival (P=0.048). Knockdown of LY6E using small interfering RNA inhibited CRC cell proliferation, migration, invasion, and soft agar colony formation, indicating some of its effects on CRC carcinogenic functions. High expression of LY6E may have oncogenic functions in CRC and be useful as a valuable prognostic marker and potential therapeutic target for CRC.

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Introduction

Colorectal cancer (CRC) occurs in the large intestine and is the third most common cancer worldwide. CRC is the second leading cause of cancer-related death (1). In addition, the recurrence rate of CRC after curative resection is 30-50% (1,2). The survival and mortality rates of patients with CRC are closely related to cancer recurrence and metastasis. In the United States, the 5-year relative survival rate of patients with CRC ranges from 90% in patients diagnosed with local disease to 14% in patients diagnosed with distant-stage disease (3). Prediction of CRC recurrence and metastasis is key for determining treatment methods for patients with CRC. However, reliable prognostic molecular biomarkers for evaluating CRC recurrence and metastasis are lacking. Thus, despite progress made towards the discovery of novel therapeutic methods, the mortality of patients with CRC remains relatively high.

Lymphocyte antigen 6 (LY6) family (LY6D, LY6E, LY6H and LY6K) is found in human lymphoid cells (4-6) and participates in T cell activation and the function of the complement membrane attack complex (7). Members of the LY6 family are low-molecular-weight glycoproteins with conserved cysteine residues bound to the cell membrane via a C-terminal glycosylphosphatidylinositol moiety. Although the biological function of LY6 family is not well-understood, these proteins are involved in signaling and cell activation (8,9). The LY6 family is associated with common copy number gain mutations in various types of cancer (10,11). Recently, numerous researchers have shown interest in the LY6 family, which plays a multifaceted role in cancer pathogenesis, stem cell maintenance, and immune regulation and its association with aggressive and intractable cancers. In particular, the mRNA expression levels of several genes in the LY6 family were significantly higher in lung, brain, breast, and head and neck cancers than in normal tissues (4,12). Lymphocyte antigen 6 complex, locus E (LY6E) is a member of the LY6 superfamily in lymphostromal cells (13-15). LY6E is a glycosylphosphatidylinositol-anchored cell-surface protein that regulates the proliferation, oncogenesis, differentiation, immunological regulation, and activation of T-lymphocytes (16). Previous studies identified LY6E as a common surface marker in gastric

and pancreatic cancer (12,17). Additionally, LY6E is associated with tumor immune escape and drug resistance in breast cancer (16). However, the expression of LY6E in lymphostromal cells of cancer tissues has not been reported so far.

In the present study, the effects of LY6E downregulation on cellular function in CRC cell lines were evaluated. Using siRNA-based technology, some roles of LY6E protein in CRC carcinogenesis were investigated. The clinical data of the present study suggested that LY6E is a prognostic marker for CRC.

Materials and methods

Immunohistochemistry and data analysis. Archived CRC tissues were obtained from 100 patients in 2005 and another 10 samples were obtained in 2018. Written informed consent was obtained from all patients for use of the specimens. The present study was approved (approval no. 2018-07-061) by the Institutional Review Board of Soonchunhyang University Cheonan Hospital (Cheonan, Korea). Immunohistochemistry (IHC) for LY6E was performed on paraffin-embedded tissues using 4-μm tissue sections mounted on slides. For immunohistochemistry staining of LY6E, all slides were blocked with serum and incubated with the LY6E primary antibody at 4˚C, overnight (1:200; cat. no. GTX101567; GeneTex, Inc.). The slides were washed and then incubated with EnVision+ System- HRP-labelled polymer anti-rabbit (cat. no. K4002; Dako; Agilent Technologies, Inc.) for 30 min at room temperature. To evaluate the immunohistochemistry results, the antibodies were stained with 70 μl 3'-3-diaminobenzidine (DAB; cat. no. K3468; Dako; Agilent Technologies, Inc.) and visualized under a light microscope. The expression of LY6E in CRC tissues was scored in a blinded manner by independent investigators, and a consensus score was determined for each specimen. The expression scoring of LY6E was graded on a four-point scale based on the intensity and percentage of LY6E staining: The percentage of staining was scored as follows: 0, 0-5; 1, 5-25; 2, 25-50; 3, 50-75; and 4, 75-100%, whereas intensity of staining was scored in the following manner: 0, negative; 1, weak; 2, moderate; 3, strong. The final scores were calculated by multiplying of two scores: 0 points for negative, 1-3 points for weak, 4-6 points for moderate and 7-12 points for strong. The LY6E low expression group included negative and weak categories, and the LY6E high expression group included cases that scored above 4 points.

Cell culture. Human CRC cell lines (SW480, SW620, HCT116 and HT29) were provided by the Korean Cell Line Bank (Seoul, Korea) and cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium (cat. no. SH30027.01; Hyclone; Cytiva) containing 10% fetal bovine serum (FBS; cat. no. 35-015-CV; Corning, Inc.) and 1% penicillin/streptomycin (cat. no. 30-022-CI; Corning, Inc.) at 37˚C in a 5% CO_2 incubator. The identities of CRC cells were confirmed by short tandem repeat (STR) profiling by KCLB and all cell lines were each purchased within the last 5 years.

Transfection of small interfering RNA. CRC cell lines were transfected with 100 nM of the appropriate small interfering RNA (siRNA) with 12 μl of HiPerFect Transfection Reagent (cat. no. 301705; Qiagen GmbH) in 12-well plates. After 72 h, the cells were used for experiments. The targeting sequences for LY6E-specific siRNA were siRNA1: 5'-GCAUUGGGA AUCUCGUGACTT-3', siRNA2: 5'-GCUUCUCCUGCU UGAACCATT-3', and siRNA3: 5'-GCCAGAGCUUUCUGU GCAATT-3'. Non-targeting control siRNAs of AccuTarget™ Negative Control siRNA (cat. no. SN-1002; Bioneer Corporation) was used as control. The LY6E-specific siRNAs were used in combination.

Reverse transcription-quantitative (RT-q) PCR. Total RNA from CRC cell lines was extracted from the control group and LY6E-siRNA treated group using RiboEx Total RNA Solution (cat. no. 301-001; GeneAll Biotechnology). Using 1 μg of the extracted RNA, cDNA was synthesized using the ReverTra Ace™ qPCR RT kit (cat. no.FSQ-101; Toyobo Life Science) according to the manufacturer's instructions. The PCR reaction temperature conditions were followed by 35 cycles of pre-denaturation (95˚C, 10 min), denaturation (95˚C, 30 sec), annealing (55-65, 15 sec) and extension (72˚C, 15 sec). RT-qPCR for LY6E was performed using primers purchased from Bioneer Corporation. To confirm the expression of genes for cancer metastasis-related cell growth and proliferation-related phenotypes, primers were designed for CDKN2A, IGF1, CXCR4 and MET and determined by RT-qPCR, and GAPDH was used as an endogenous control (Table I). The target gene mRNA level was quantified as GAPDH mRNA, and the gene expression level was analyzed using the $2^{-\Delta\Delta Cq}$ method (18). The amplicon was mixed with 10X loading buffer (TaKaRa Bio, Inc.) and then agarose gel electrophoresis was performed using 2% agarose gel containing NEOgreen (NeoScience) DNA staining reagent. then FluoroBox (Neoscience) nucleic acid gel imaging system was used to visualize the amplicon, and the mRNA expression level was analyzed using image J 1.53t software (National Institutes of Health).

Western blotting. Total cellular protein was extracted using PRO-PREP (Intron Biotechnology, Inc.) and quantified using a BCA kit (cat. no. 23227; Thermo Fisher Scientific, Inc.) and an xMark Microplate Absorbance Spectrophotometer (Bio-Rad Laboratories, Inc.). The 30 μg protein was analyzed using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and β-actin was used as a loading control. Then proteins were transferred to a polyvinylidene fluoride membrane (cat. no. ISEQ00010; MilliporeSigma). The polyvinylidene fluoride membrane was incubated with anti-LY6E antibody (1:100; cat. no. ABIN517581; Antibodies-online GmbH) overnight at 4˚C, and the appropriate horseradish peroxidase-conjugated secondary antibody (1:2,000; cat. no. 32260; Thermo Fisher Scientific, Inc.) was reaction for 2 h at room temperature. Protein was visualized with the addition of enhanced chemiluminescence (cat. no. 34577; Thermo Fisher Scientific, Inc.). Western blot results were visualized using a Molecular Imager ChemiDoc XRS + system (Bio-Rad Laboratories, Inc.) and quantified using the ImageJ software (National Institutes of Health).

Migration and invasion assay. The control group and LY6E-siRNA treated group of cells were collected, and $2x10^5$ cells were resuspended in 200 μl of medium and

Table I. Primer sequences were used in reverse-transcription polymerase chain reaction.

Gene name	Amplicon size (bp)	Primer sequence (5'à3')
LY6E	391	Sense: AAGTAGCTGACCACAGAGCA Antisense: TGTCACGAGATTCCCTGCAT
CDKN2A	384	Sense: TCTGACCATTCTGTTCTCTC Antisense: CTCAGCTTTGGAAGCTCTCA
IGF1	134	Sense: CTCTTCAGTTCGTGTGTGGAGAC Antisense: CAGCCTCCTTAGATCACAGCTC
CXCR4	127	Sense: CTCCTCTTTGTCATCACGCTTCC Antisense: GGATGAGGACACTGCTGTAGAG
MET	142	Sense: TGCACAGTTGGTCCTGCCATGA Antisense: CAGCCATAGGACCGTATTTCGG
GAPDH	131	Sense: CCAGCCGAGCCACATCGCTC Antisense: ATGAGCCCCAGCCTTCTCCAT

LY6E, lymphocyte antigen 6 complex locus E.

inserted into the upper of 6.5 mm Transwell® with 8.0 μm Pore Polycarbonate Membrane Insert with serum-free RPMI-1640 medium. In the lower chamber, 600 μl of supplemented medium was added, and the cells were cultured at 37˚C in a humidified incubator for 48 h. The remaining cells in the upper chamber that could not pass through the 8 μm pore-size filter were removed using a cotton swab. The cells were fixed with 3.7% neutral-buffered formalin at room temperature for 3 min and stained with 0.005% crystal violet at room temperature for 20 min. Cell images were obtained, and cells were counted using an inverted microscope. Invasion assays were performed as aforementioned for the migration assay, except that the Transwell chamber was coated with 50 μl Matrigel 3 h before the experiment and stored at 37˚C.

Proliferation assay (WST-1 assay). Post 48-h transfection with LY6E-inhibited and scramble control siRNA, nine replicates ($1x10^4$ cells/well) were uniformly dispensed in a 96-well plate and were incubated at 37˚C, 5% CO_2 for 24, 48 and 72 h. Next, WST-1 assay reagent (EZ-CYTOX; cat. no. EZ-1000; DogenBio; http://www.dogenbio.com/) was added 10 μl (5 mg/ml) at every set time and reacted for 1 h. The absorbance was measured at 562 nm using a microplate reader.

Soft agar colony formation assay. As basement agar, 0.5% agarose was prepared in RPMI-1640 medium supplemented with 10% FBS. As top agar, the control and LY6E-siRNA treated groups were plated in six-well dishes ($5x10^3$ cells/well) in 0.35% agarose in RPMI-1640 supplemented with 10% FBS. After 10-14 days, the colonies were stained for 1 h at room temperature with 0.005% crystal violet and images were captured after removing the growth medium. Numbers of colonies with a diameter greater than 30 μm were quantified after 2 weeks and analyzed using Image J 1.53t software (National Institutes of Health).

Statistical analysis. The data are presented as the average of the values of three replicates. Analysis was conducted using SPSS v19 software (IBM Corp.) was used for statistical analysis using unpaired Student's t-test. Mann Whitney U test and Kruskal-Wallis followed by Dunn's or Steel-Dwass post hoc tests were used to compare for analyzing the IHC scores. Cox regression and Kaplan-Meier curves were used to analyze the associations between LY6E expression and patient outcomes. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

LY6E expression and survival analysis in patients with CRC. To investigate the genes affecting among the LY6 family in CRC, the expression of LY6 family (LY6D, LY6E, LY6H, LY6K) was confirmed in colon adenocarcinoma and rectal adenocarcinoma based on the The Cancer Genome Atlas database (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga). As a result, it was confirmed that only LY6E was specifically overexpressed among LY6 family in CRC and genes other than LY6E were rarely expressed in CRC (Fig. 1A). The mRNA expression was analyzed from 275 colon cancer tissues, 349 relevant normal tissues, 92 rectal cancer tissues, and 318 normal rectum tissues from the Gene Expression Profiling Interactive Analysis (GEPIA) website (http://gepia.cancer-pku.cn/index.html) to investigate the role of LY6E in CRC. LY6E was found to show higher mRNA expression in tumor tissues than in normal tissues (Fig. 1B and C). Immunohistochemical staining was performed using tissues from 110 patients obtained from Soonchunhyang University Cheonan Hospital. Low LY6E expression was detected in 78 (70.9%), while high LY6E expression was found in 32 (29.1%) CRC tissues (Fig. 1D). However, lymphostromal cells did not express LY6E as revealed by immunohistochemistry. High LY6E expression was significantly associated with pN ($P<0.0001$) and clinical stage ($P<0.0001$, Fig. 1E and F, Table II). Univariate and multivariate cox proportional hazard regression analysis were performed to further evaluate according

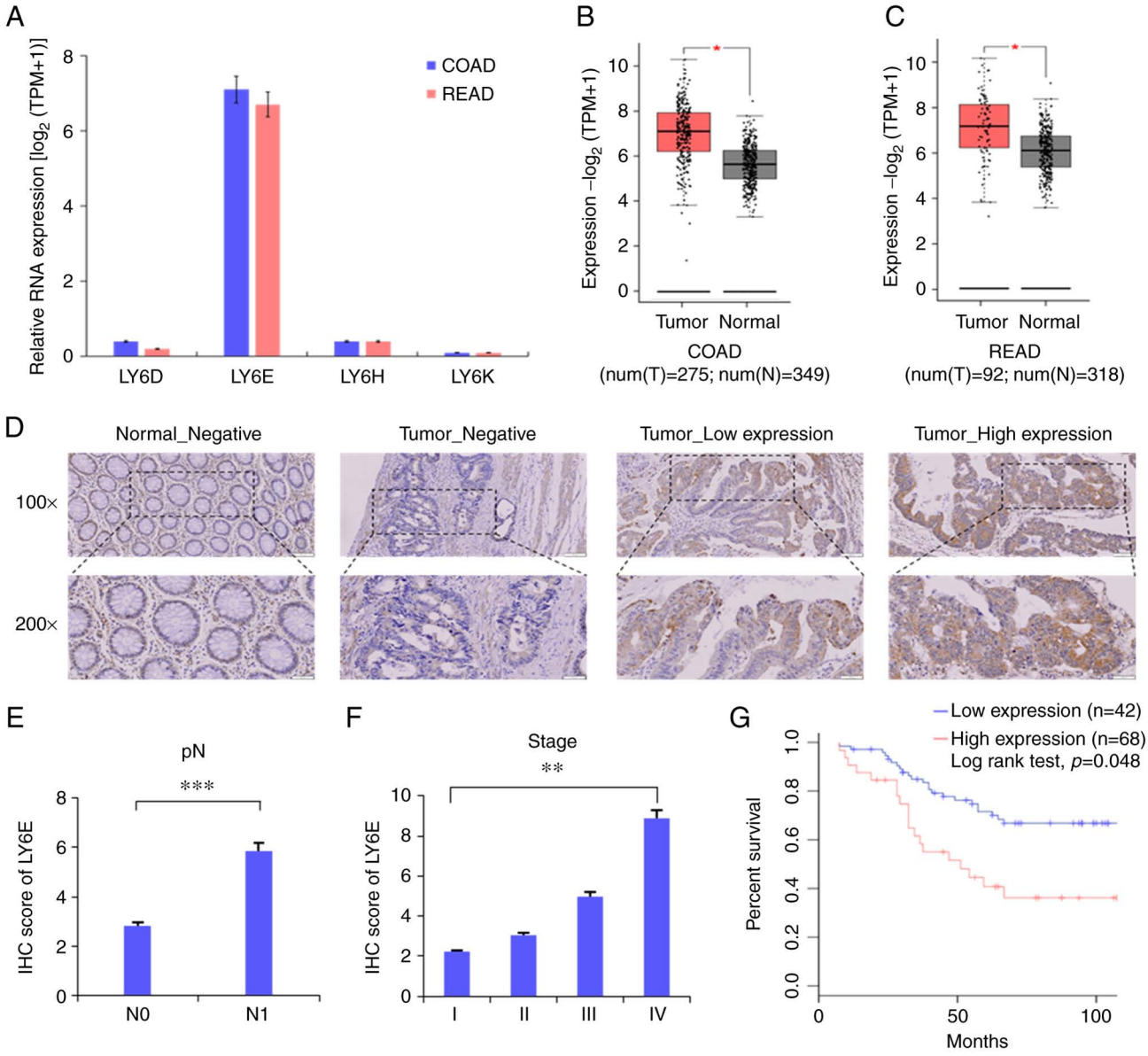

Figure 1. LY6E is upregulated in colorectal cancer and high expression level of LY6E is associated with poor prognosis. (A) Expression of LY6E and other lymphocyte antigen 6 family in CRC. Comparison of LY6E expression in tumor and normal tissue of (B) colon and (C) rectum. (D) Representative immunohistochemical staining images of LY6E expression in human CRC and normal tissues (Scale bar, 100 μm in 100X, 50 μm in 200X). (E and F) IHC scores of LY6E according to pN and clinical stage were evaluated in Mann-Whitney analysis and Kruskal-Wallis test. Error bar shows standard deviation. (G) Overall survival rates of patients with CRC according to LY6E expression level were evaluated by Kaplan-Meier and log-rank test. Log-rank tests were used to compare the overall survival between two groups (P=0.048). *P<0.05, **P<0.01 and ***P<0.001. LY6E, lymphocyte antigen 6 complex locus E; CRC, colorectal cancer.

to clinical pathological factors whether expression of LY6E can be used as independent predictive biomarkers to predict treatment response. Univariate and multivariate analysis were expressed using hazard ratio (HR) according to clinicopathological factors (Table III). The univariate HR values for vascular invasion were 2.622 (1.151-5.974, P=0.022), and the univariate and multivariate HR values for lymphatic invasion were 3.216 (1.683-6.144, P<0.001), 2.272 (1.019-5.066, P<0.001), and metastasis were 6.860 (2.800-16.804, P<0.001), 6.472 (2.252-18.594, P<0.001). Also, in a Kaplan-Meier analysis of 110 patients, high LY6E expression was significantly associated with overall survival. High expression of LY6E significantly reduced cancer-specific survival in patients compared with those with low LY6E expression (log-rank test; P=0.048, Fig. 1G).

LY6E expression in CRC cells is reduced by siRNAs. The expression of LY6E was confirmed using RT-qPCR and immunoblotting in four CRC cell lines (SW480, SW620, HCT116 and HT29). Then, four CRC cell lines were transfected by LY6E-targeted siRNA to inhibit LY6E expression. The mRNA level of LY6E decreased significantly in SW480 (57.8±4.6%), SW620 (72.7±2.5%), HCT116 (58.9±5.3%), and HT29 (50.9±4.6%) cells compared with that in the control group (Fig. 2A and B, P<0.001). Additionally, the protein level of LY6E decreased in SW480 (63.9±3.9%), SW620 (78.5±3.3%), HCT116 (55.3±3.2%) and HT29 (69.3±7.2%) cells compared with that in the control group (Fig. 2C and D, P<0.001). These results indicated that LY6E expression was successfully reduced at both the mRNA and protein levels in CRC cells.

Table II. Comparison of clinicopathological factors and LY6E expression in patients with CRC by chi-square analysis.

Clinicopathological factors	Expression level of LY6E		Total (n=110)	P-value
	Low (n=42)	High (n=68)		
Age, years (mean ± SD)	62.1 (13.5)	63.2 (13.9)	62.5 (13.5)	
Sex, N (%)				0.686
Female	17 (41.5)	24 (58.5)	41 (37.3)	
Male	25 (36.2)	44 (63.8)	69 (62.7)	
pT stage, N (%)				0.765
1, 2	5 (41.7)	7 (58.3)	12 (10.9)	
3, 4	37 (37.8)	61 (62.2)	98 (89.1)	
pN stage, N (%)				<0.001
0	27 (57.4)	20 (42.6)	47 (42.7)	
1, 2	15 (23.8)	48 (76.2)	63 (57.3)	
Vascular invasion, N (%)				0.241
Negative	39 (40.6)	57 (59.4)	96 (87.3)	
Positive	3 (21.4)	11 (78.6)	14 (12.7)	
Lymphatic invasion, N (%)				0.091
Negative	37 (42.5)	50 (57.5)	87 (79.1)	
Positive	5 (21.7)	18 (78.3)	23 (20.9)	
Metastasis, N (%)				0.081
Negative	42 (40.4)	62 (59.6)	104 (94.5)	
Positive	0 (0.0)	6 (100.0)	6 (5.5)	
Clinical stage, N (%)				<0.001
I and II	27 (60.0)	18 (40.0)	45 (40.9)	
III and IV	15 (23.1)	50 (76.9)	65 (59.1)	

LY6E, lymphocyte antigen 6 complex locus E; CRC, colorectal cancer.

Table III. Cox regression analysis of the clinicopathological factors in colorectal cancer.

Clinicopathological factors	Variable	Univariate analysis		Multivariate analysis	
		HR (95% CI)	P-value	HR (95% CI)	P-value
Age, years	<60 vs. ≥60	0.921 (0.479-1.773)	0.964	0.984 (0.477-2.028)	0.806
Sex	Female vs. male	1.154 (0.614-2.170)	0.436	1.315 (0.660-2.618)	0.657
pT stage	T1, T2 vs. T3, T4	0.532 (0.236-1.200)	0.038	0.405 (0.172-0.949)	0.128
pN stage	N0 vs. N1, N2	1.812 (0.960-3.418)	0.196	3.264 (0.544-19.599)	0.066
Vascular invasion	Negative vs. Positive	2.622 (1.151-5.974)	0.365	1.594 (0.582-4.364)	0.022
Lymphatic invasion	Negative vs. Positive	3.216 (1.683-6.144)	0.045	2.272 (1.019-5.066)	<0.001
Metastasis	Negative vs. Positive	6.860 (2.800-16.804)	<0.001	6.472 (2.252-18.594)	<0.001
Clinical stage	I, II vs. III, IV	1.737 (0.920-3.280)	0.326	0.394 (0.061-2.525)	0.088
Expression level of LY6E	Low vs. High	1.912 (0.991-3.692)	0.397	1.385 (0.652-2.944)	0.053

LY6E, lymphocyte antigen 6 complex locus E; HR, hazard ratio; CI, confidence interval.

Downregulation of LY6E results in reduced tumorigenicity, migration and invasiveness of CRC cells. The effect of LY6E downregulation on cell migration and invasion was examined. LY6E downregulation notably reduced cell migration into the lower chamber compared with that of control cells (SW480, 64.3±8.2%; SW620, 73.5±5.3%; HCT116, 58.3±7.9%; and HT29, 52.1±8.0%) (Fig. 3A and B, P<0.001). siRNA_LY6E cells had significantly reduced invasive properties (SW480, 57.3±7.3%; SW620, 69.3±8.4%; HCT116, 76.7±7.7%; and HT29, 55.4±7.0%) (Fig. 3C and D, P<0.001). To confirm

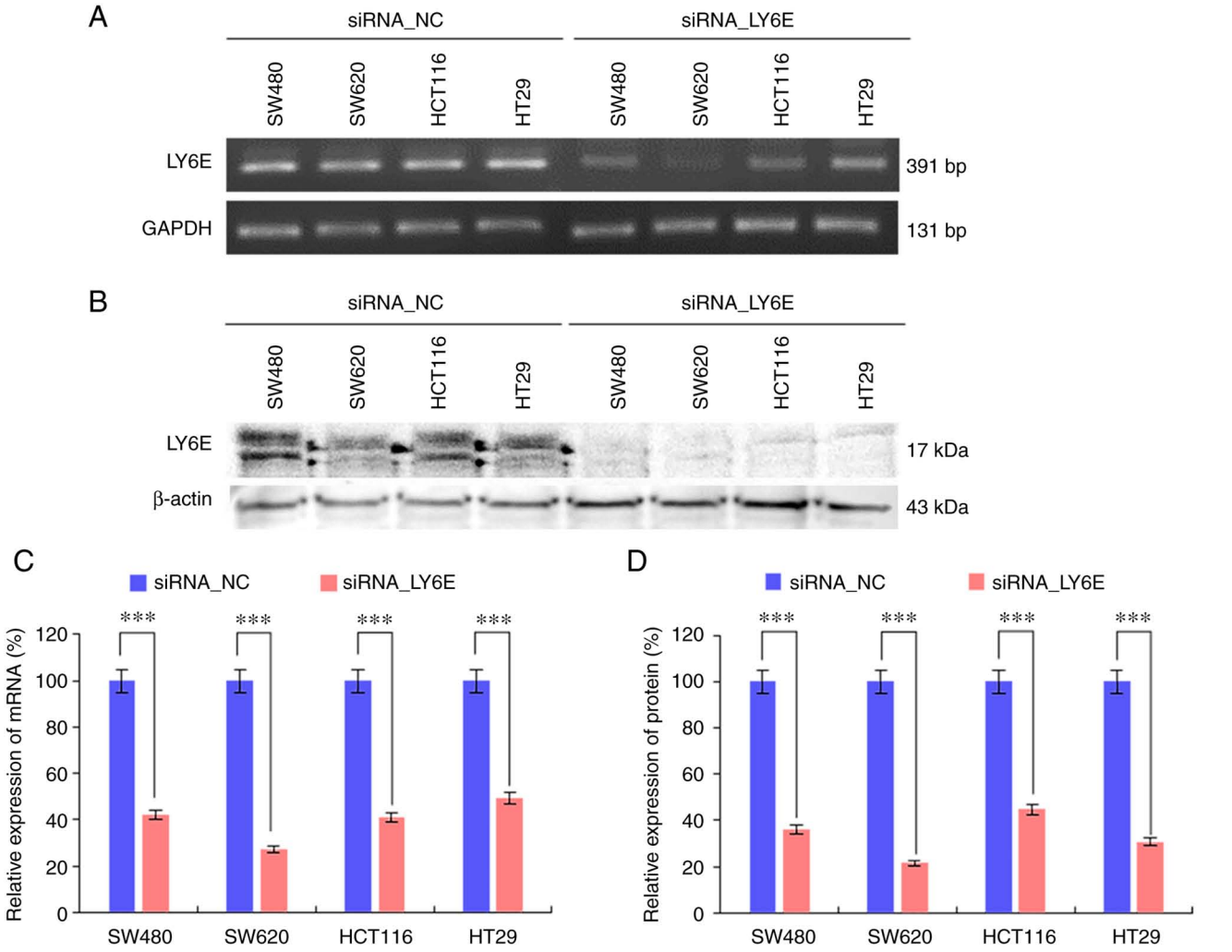

Figure 2. LY6E mRNA and protein level is downregulated by siRNA targeting LY6E. SW480, SW620, HCT116 and HT29 cells were transfected with either negative control or LY6E_siRNA. (A) RT-qPCR was performed to confirm LY6E expression in CRC. (B) Relative expression of LY6E mRNA. (C) Expression levels of LY6E in CRC were confirmed by immunoblotting. (D) Relative expression of LY6E protein. GAPDH and β-actin were used as the loading control for RT-qPCR and immunoblotting. Error bar shows standard deviation. ***P<0.001. LY6E, lymphocyte antigen 6 complex locus E; siRNA, small interfering RNA; CRC, colorectal cancer; RT-qPCR, reverse transcription quantitative PCR; NC, negative control.

whether LY6E plays an important role in CRC cell colonization, a colony formation assay was performed. Cells in which LY6E was downregulated showed decreased colony formation compared with the control group (SW480, 65.3±14.7%; SW620, 79.2±10.6%; HCT116, 69.5±9.7%; and HT29, 79.8±8.0%) (Fig. 3E and F, P<0.001).

Inhibition of the LY6E reduces cell growth and proliferation. Proliferation assay (WST-1 assay) was performed to determine whether the decreased LY6E gene is involved of cell proliferation in CRC. Cells in which LY6E was downregulated showed significant absorbance values compared with the control group (SW480, 1.81±0.09; SW620, 1.72±0.11; HCT116, 1.86±0.07; and HT29, 1.50±0.12) (Fig. 4A-D, P<0.001). The expression of genes for phenotypes related to cell growth and cell proliferation was compared between CRC cells in which LY6E expression was suppressed and a control. MET, IGF1, CDKN2A and CXCR4 are cell growth and cell proliferation-related genes that affect cancer metastasis (19) and were verified using RT-qPCR. Expression of MET, IGF1, CDKN2A, and CXCR4 was confirmed in four CRC control cell lines. By contrast, LY6E-suppressed CRC cells were confirmed that the expression of MET, IGF1, CDKN2A and CXCR4 was reduced (Fig. 4E and F).

Discussion

Although CRC treatment and diagnostic methods have improved over the past few decades, the survival rate of patients with stage IV CRC remains low. The survival rate of these patients depends on the presence of lymph nodes metastasis, distant metastasis and recurrence. In the Republic of Korea, the survival rate of patients with CRC with regional lymph node metastasis is 82.6% but is remarkably decreased by 20.2% in patients with CRC with distant metastasis (1). Moreover, the prediction of CRC recurrence is essential for determining chemotherapeutic or targeted molecular therapeutic approaches. Several biomarkers correlated with CRC progression have been identified in molecular studies. The efficacy of conventional therapies for CRC is increased by targeted therapies. Notably, prognostic biomarkers can help oncologists optimize therapies for challenging cases of CRC.

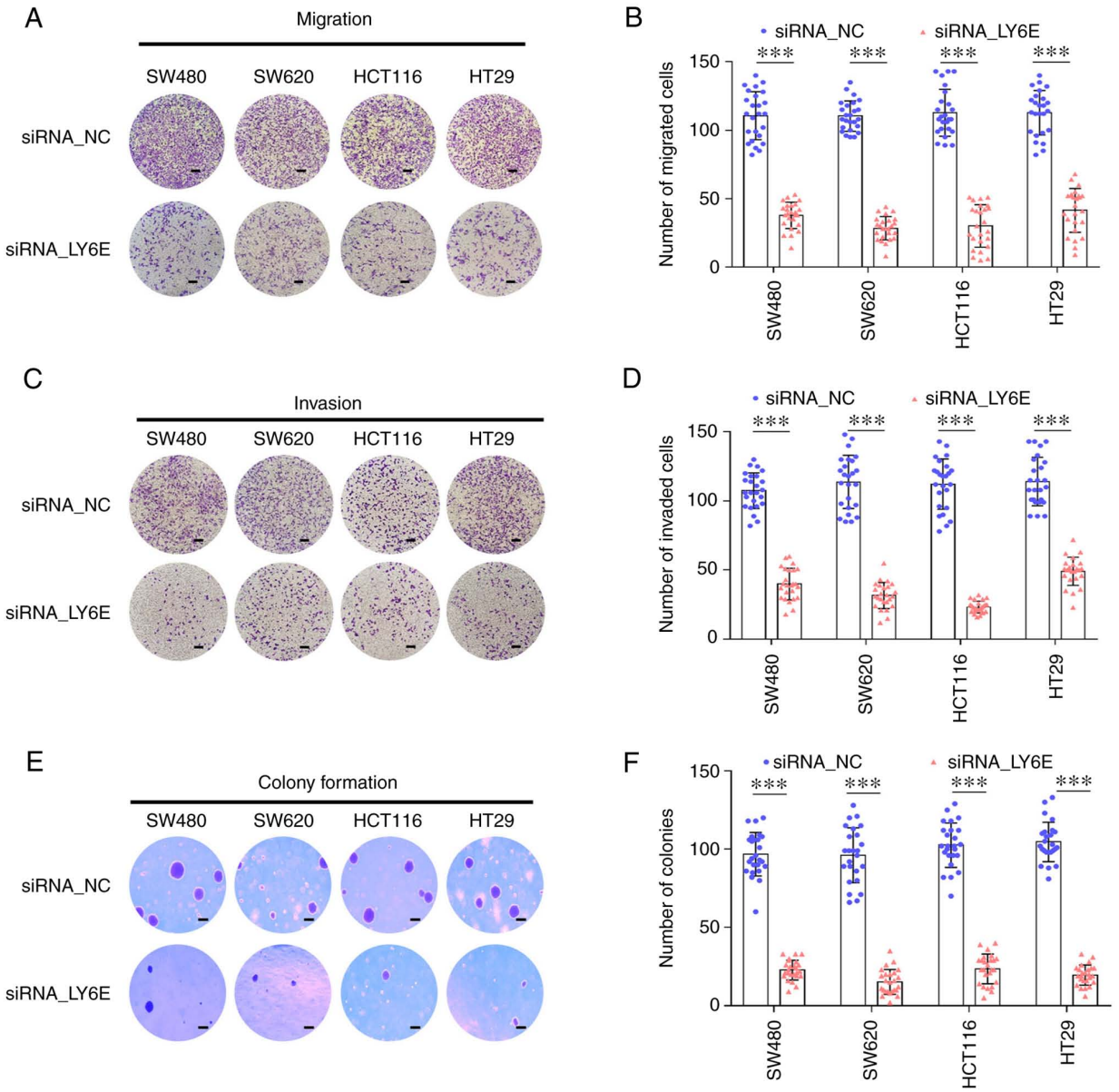

Figure 3. Downregulation of LY6E expression results in reduced migration, invasion and colony formation abilities. (A) Representative Transwell migration assay images of negative control and LY6E siRNA-treated CRC cells (magnification, x40). (B) Based on five random fields, the average number of migratory cells were shown. (C) Representative images of invasion assay of CRC cells according to LY6E expression (magnification, x40). (D) Difference in the number of invasive cells by LY6E downregulation were shown. (E) Representative images of colonies were shown (magnification, x40). (F) Number of colonies from each colorectal cancer cell line were quantified. Error bar shows standard deviation. ***P<0.001. LY6E, lymphocyte antigen 6 complex locus E; CRC, colorectal cancer; siRNA, small interfering RNA; NC, negative control.

In T-cell among lymphocytes, LY6 functions to regulate proliferation and differentiation, and immunological regulation and activity (19). In addition, LY6E affects the development of cancer by increasing CTLA4 and tumor-infiltrating T regulatory cells (16). LY6E has been reported to play a multifaceted role in cancer pathogenesis, stem cell maintenance, immunomodulation and association with aggressive and refractory cancers (4,13,16,20). In breast cancer, it is related to immune evasion and anticancer drug resistance (16). In particular, LY6E expression in CRC plays important roles in CRCs by increasing the expression of PDL1 and has been reported as a biomarker (16). The present *in vitro* studies revealed that LY6E is related to increased cellular proliferation, invasion, and migration, which are hallmarks of tumorigenesis. AlHossiny *et al* (16) reported that high expression of LY6E is associated with TGF-β signaling maintenance in breast cancer. TGF-β signaling is involved in cancer cell growth, metastasis, invasion, and epithelial-to-mesenchymal transition (EMT) (20-24) and regulates chemotherapeutic drug resistance (25,26). However, Yeom *et al* (13) demonstrated that LY6E downregulates PTEN expression, thereby activating PI3K-AKT signaling in gastric cancer. Knockdown of LY6E inhibits the expression of ZEB1, an EMT inducer and E-cadherin (27). In the present study, LY6E overexpression was an independent prognostic marker related to poor overall survival of patients with CRC. It remains unclear whether LY6E regulates TGF-β or PI3K-AKT signaling and other signaling in CRC carcinogenesis. Therefore, further studies are needed

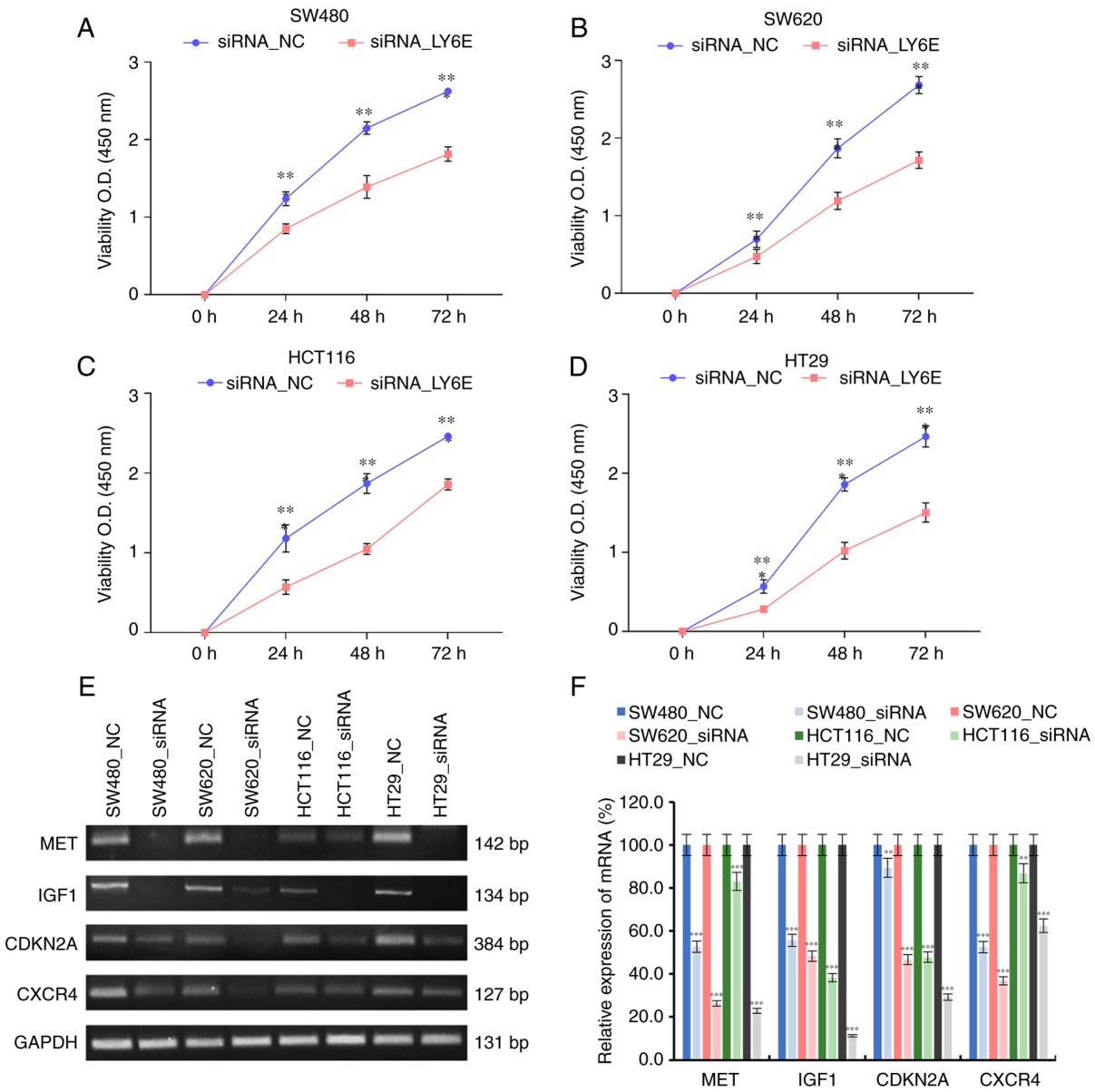

Figure 4. Downregulation of LY6E impairs cell growth and proliferation in CRC cell lines. (A-D) Cell proliferation of negative control cells and LY6E siRNA-transfected cells were compared over time. (E) Reverse transcription-quantitative PCR was performed to confirm cell growth and cell proliferation related genes expression by negative control and LY6E siRNA. (F) Relative cell growth and proliferation-related genes expression in CRC cell lines. Error bar shows standard deviation. *P<0.05, **P<0.01 and ***P<0.001. LY6E, lymphocyte antigen 6 complex locus E; CRC, colorectal cancer; siRNA, small interfering RNA; NC, negative control.

on the effects of LY6E, TGF-β and PI3K-AKT signaling in CRC and the direct correlation between LY6E and T cells. In addition, it is necessary to elucidate the relationship between the tumorigenesis and metastasis according to the expression of LY6E through *in vivo* studies.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DJ and HJK conceptualized the present study. IH, SK, SR, HK and SO conducted investigation. TSA, DHK and M-JB

curated the data. SK and IH prepared the original draft of the manuscript. DJ wrote, reviewed, and edited the manuscript and supervised the study. IH and SK performed visualization. DJ and SK confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved (approval no. 2018-07-061) by the Institutional Review Board of Soonchunhyang University Cheonan Hospital (Cheonan, Korea). Written informed consent was obtained from all patients for use of the specimens.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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